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BWL Technical Memorandum 9-8

STABILITY OF LIQUID CULTURES OF COCCIDIOIDES IMMITIS ARTHROSPORES TO STORAGE, DRYING, AND FREEZING AND THAWING

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I. SUMMARY

Storage of submerged cultures of Coccidioides immitis grown in the liquid synthetic medium of Roessler et al has not been a problem. Eighty to one hundred per cent viability can be maintained for as long as 6 months in cultures stored in their own spent medium at 5°C. This survival can be improved (particularly during extended storage beyond the 6 month interval) by replenishing the carbon and nitrogen sources after growth. Optimum physical conditions for storage obtained (a) when the air space to culture volume ratio (A/V) within the storage container was equal to 1 or less, and (b) in more concentrated cell suspensions rather than in dilutions of normal growth levels (particularly beyond the 6 month interval). Higher survival was obtained with cultures incubated for 14 days than with those incubated for either 7, 21, or 28 days. Neither shaking nor opening the storage containers at monthly intervals had an appreciable effect on survival throughout 12 months storage at 5°C.

Carbohydrates (particularly glucose) were of more value in protection of arthrospores against death due to drying than were protein compounds. Highest survival (45 to 49 per cent) resulted from resuspension (before drying) in a menstruum composed of 8 per cent aqueous glucose with small amounts of added gelatin and agar as compared with 36 per cent with glucose alone, and < 1 per cent for the controls. As suspected at the beginning of these studies, compounds protecting cells against death due to drying resulted in improved aerosol stability. Arthrospores resuspended in 8 per cent glucose before aerosolization have maintained as high as 27 per cent survival as compared with 1 per cent or less for those aerosolized from a suspension in their own supernatant liquid (data obtained from the Mycology Section, SO Division). Higher survival to drying was obtained with arthrospores incubated for 14 and 21 days, than with those 7 or 28 days old.

The temperature of freezing was more critical than the temperature of thawing in survival of arthrospores to freezing and thawing. With a constant thawing temperature of 5°C, approximately 40 per cent survival to freezing at -15°C was obtained with arthrospores suspended in their own supernatant liquid, as compared with < 1 per cent for those frozen at -72°C. Moreover 40 per cent survival to a holding temperature of -72°C could be maintained, provided the arthrospores were first frozen at -15°C before being placed at -72°C. Almost complete protection against death due to -15°C freezing was obtained by resuspension of cells in 8 per cent aqueous glucose before freezing. This treatment was much less effective with cells frozen at -72°C. Concentration of cells had no appreciable effect on survival to freezing, however, larger volumes (20 or 40 ml) of culture survived better than smaller volumes (5 or 10 ml). In general, younger cells survived to a greater extent than older cells.

A very interesting observation, noted consistently throughout the studies comprising this report, was the significant, enhancing effect of glucose on the survival to storage, drying, freezing, and also to aerosolization.

II. INTRODUCTION

Relatively little has been published about Coccidioides immitis outside of the fields of epidemiology and morphology. Moreover, most of the published work on culture methods has been concerned with growth on solid medium, rather than with submerged growth. The studies with which this report is concerned were aimed at the improvement of survival during storage, drying, and freezing and thawing. The method of approach has been through a study of the nutritional and physical environmental conditions influencing stability.

Improvement of stability to drying and/or freezing was of two-fold importance: (a) the viability of stock cultures maintained by lyophylization was found to be extremely low (< 1 per cent), and (b) it was postulated that the low aerosol stability of arthrospores aerosolized from liquid suspensions was in some way connected with poor survival to drying. Studies on the improvement of aerosol stability through application of findings on improved survival to drying were carried out with the cooperation of the Screening Branch, MB Division, the Mycology Section, SO Division, and Aerobiology Division.

III. MATERIALS AND METHODS

A. MEDIUM

The majority of these studies were carried out using the glucose, ammonium acetate, inorganic salts medium of Roessler et al[1]* which has the following composition:

Component	Molarity
Glucose	0.11
Ammonium acetate	0.08
K_2HPO_4	0.015
KH_2PO_4	0.015
$MgSO_4 \cdot 7H_2O$	0.008
$ZnSO_4 \cdot 7H_2O$	0.000062

Deviations from this medium are so noted in the results section. The components of the medium were dissolved in distilled water, with a resulting pH of 6.6 before autoclaving. Limited comparisons were made between this medium and the complex medium of Roessler et al[1] (2 per cent glucose, 1 per cent peptone, and 0.1 per cent yeast autolysate).

*See Literature Cited, page 45.

B. INOCULUM

Routinely inocula of 3×10^7 to 4×10^7 arthrospores (0.1 ml of a 14 day culture grown in the same medium as the experimental cultures) were used. Stock cultures for preparation of inocula were maintained on glucose, peptone, yeast autolysate agar slants[1] at 5°C. Fresh liquid inocula were prepared at three month intervals.

C. STRAINS

C. immitis, strain Cash was selected (from a collection of approximately 65 strains maintained in the Nutrition Branch) for these studies, due to the uniparticulate nature of its arthrospore suspensions in liquid culture which enabled accurate assay of viable cell count for determination of growth yield and storage viability, etc. This strain was isolated from a disseminated case of coccidioidomycosis and its virulence compared favorably with that of other available strains. Other strains will be indicated where used in the text.

D. GROWTH CONDITIONS

Arthrospore cultures were incubated at 34°C for 14 days (unless stated otherwise) on a reciprocating shaker with a 4½ inch stroke at 100 excursions per minute. The cultures were grown in 250 ml Erlenmeyer flasks (50 ml of medium per flask) closed with rubber stoppers bearing thistle tubes packed with cotton. This type of closure provided adequate aeration for maximum growth in the media used and was used in preference to cotton plugs for safety reasons.

E. ASSAY METHODS

1. Viability

Viable counts in fresh and treated cultures were determined by the pour-plate method of counting, using a plating medium composed of 2 per cent glucose, 1 per cent peptone, 0.1 per cent yeast autolysate, and 2 per cent agar[1]. Appropriate dilutions of the cultures in tryptose saline diluent (0.1 per cent tryptose and 0.5 per cent NaCl in tap water) were used to inoculate the plates, which were incubated for 48 hours at 34°C and an additional 16 hours at room temperature before counting.

A statistical analysis (Goldschmidt, 1955[2]) to determine variability of viable cell assay among cultures and among plate counts using 1 to 12 flasks and 1 to 3 pour plates per flask indicated an accuracy of ± 3.4 per cent with 95 per cent confidence limits when 3 plates were used for each of 3 replicate flasks.

Additional values (figures in parentheses) are given in table II (results of a typical long-term experiment) to indicate the upper and lower limits of values used in calculating the mean.

2. Morphology

The morphological development of the cultures was determined by microscopic examination of wet-mounts of culture material suspended in lactophenol blue solution (aqueous solution of 20 per cent, each, lactic acid and phenol; 40 per cent glycerol; and 0.05 per cent cotton blue stain).

F. STORAGE CONDITIONS

Unless stated otherwise, duplicate 14 day arthrospore cultures were pooled and stored in liquid suspension at 5°C in 8 ounce Duraglass prescription bottles (100 ml per bottle) closed with rubber-lined screw caps. The bottles were stored under static conditions and were undisturbed between the monthly intervals at which viability determinations were made.

G. DRYING METHODS

Ten ml volumes of arthrospore cultures were added to 50 ml centrifuge tubes containing twelve 5 mm glass beads and centrifuged at 2000 rpm for 1 hour. Spent supernatant medium was removed by aspiration and the packed cells were resuspended in the various drying menstrua to be tested. The centrifugation and removal of supernatant liquid was repeated. An even distribution of the packed cell paste over the inside surface of the centrifuge tubes was achieved through movement of the glass beads by rotation of the tubes. The tubes were then placed over calcium sulfate in a desiccator and the air removed by means of a vacuum pump. The evacuated desiccator was placed at 5°C for one week. The dried cultures were resuspended in their original volume with either fresh synthetic medium or tryptose saline diluent, and survival was determined by viable plate counts.

H. FREEZING AND THAWING METHODS

Arthrospore cultures in 50 ml pyrex glass centrifuge tubes (10 ml per tube) were placed in a deep freeze chest at -15°C or submerged in an acetone-dry ice bath at -72°C and frozen en masse. The frozen cultures were placed in the cold room at 5°C, or in incubators at 25°C or 37°C for thawing. Survival following freezing and thawing cycles was determined by viable plate counts.

IV. EXPERIMENTAL RESULTS

A. STORAGE

1. Medium, Temperature and Strains

It was noted that liquid suspensions of C. immitis arthrospores grown and stored in Roessler's synthetic medium remained viable after two years storage at 5°C, whereas those grown and stored in complex medium (glucose, peptone, yeast autolysate) were nonviable.

a. Viability at 5°C and 25°C

Preliminary studies compared the growth media, storage temperatures, pre-storage supplements, and the strains shown in table I. Three replicate cultures (50 ml per culture) of C. immitis, strains Cash or M-11 (a rodent isolate from the state of Arizona) were placed in storage after the following treatment: (a) untreated (controls); (b) centrifuged and resuspended to the original volume in the corresponding fresh growth medium; (c) supplemented immediately preceding storage with a final level of 2.0 per cent glucose; and (d) supplemented immediately preceding storage with a final level of 0.25 per cent yeast autolysate.

As seen in table I, the main factors affecting viability during storage were the growth medium and the temperature of storage. The addition of glucose to the cultures immediately before storage, enhanced the survival to three months storage. Although this effect was not pronounced, it was consistent and occurred in a majority of the cultures of both strains grown in synthetic medium and stored at either 5°C or 25°C. The addition of yeast autolysate had a deleterious effect on the viability of stored cultures. Cultures grown in the synthetic medium maintained distinctly higher viabilities at each storage interval, and the detrimental effect of the 25°C storage temperature was not as pronounced as in cultures grown in the complex medium.

b. Effect of Supplementation of Cultures Immediately Before Storage

(1) Strain Cash, Grown in Roessler's Synthetic Medium

It was shown[3/] that the ammonium acetate and glucose content of the medium was exhausted during growth of C. immitis by approximately the 9th and 20th day of incubation respectively. This information, and data in table I concerning enhancement of storage viability by supplements of glucose to the cultures, prompted further investigation of the influence of supplementing the cultures before storage.

Replicate cultures of the Cash strain were grown in synthetic medium for 21 days (to assure complete exhaustion of the glucose and ammonium acetate content of the medium). Triplicate 100 ml aliquots of the pooled cultures were supplemented with the original levels of ammonium acetate (nitrogen source), glucose (carbon source), or the two in combination, with three left unsupplemented as controls. All cultures were stored at 5°C in 8 oz prescription bottles and the viability determined at monthly intervals.

As shown in table II, survival of all cultures was 100 per cent or more throughout 10 months storage. The glucose supplement was more beneficial to increased survival than the ammonium acetate supplement, and the effects of the two compounds were additive when used in combination.

c. Effect of Drying and Resuspending in 8 Per Cent Glucose on Survival at 5°C

Replicate cultures of C. *limitis* were centrifuged for 1 hour at 2000 rpm and: (a) resuspended in their own supernatant liquid as controls, (b) resuspended in 8 per cent aqueous glucose*, or (c) resuspended in 8 per cent glucose and dried for one week in an evacuated desiccator over calcium sulfate following removal of the supernatant liquid, and again resuspended in 8 per cent glucose. All cultures were placed in storage at 5°C and viability assayed at monthly intervals.

As indicated in table III, the control cultures and the undried cultures resuspended in 8 per cent glucose maintained normal survival of approximately 100 per cent throughout 6 months storage. However, cultures subjected to drying before wet storage at 5°C, exhibited a steady decrease in survival to 33 per cent at the end of 6 months, thus indicating a detrimental effect of the drying process on storage stability of the organism.

In order to re-examine the detrimental effect of drying on storage stability at 5°C, replicate resuspended cultures from other drying experiments (testing various drying menstrua) were held for 30 days at 5°C and assayed for viability. Using the plate counts obtained immediately after drying as a base line for 100 per cent recovery, marked decreases in viability (44 to 99 per cent) were noted after 30 days storage at 5°C, thus corroborating the data in table III.

d. Viability at -15°C

(1) Effect of Temperature

Two series of cultures were grown under standard conditions for 21 days, placed in 50 ml pyrex glass centrifuge tubes (10 ml per tube) and centrifuged at 2000 rpm for 1 hour. One series was resuspended in its own supernatant liquid and the other in 8 per cent aqueous glucose. All cultures were frozen at -15°C. Six cultures (three from each series) were immediately placed at 5°C to thaw. In order to differentiate between death due to freezing (and/or thawing) and that due to storage at -15°C, viable counts were made on these cultures to establish a survival base line from which the viable counts during storage were expressed as per cent viability. The remaining cultures were left at -15°C. Three replicates from each series were removed from storage at monthly intervals, thawed at 5°C, and assayed for viability.

As noted in table IV, survival of cultures, in the absence of added glucose, decreased to less than 50 per cent at the end of 1 month storage at -15°C, and progressively decreased to 14 per cent at 6 months. Improved survival was noted in cultures resuspended in 8 per cent glucose before freezing (77 and 53 per cent at 1 and 6 months, respectively). However, in neither instance was the survival at -15°C equal to the normal survival of cultures stored at 5°C (approximately 100 per cent at 6 months).

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*See section on Drying for explanation of the use of 8 per cent aqueous glucose.

(2) Effect of the Freezing Process

To determine whether the low temperature of storage, or the actual process of freezing and thawing resulted in the sub-standard storage stability at -15°C, replicate arthrospore cultures were stored at 5°C following (a) no treatment, (b) resuspension in 8 per cent aqueous glucose, and (c) resuspension in 8 per cent glucose followed by freezing at -15°C, and thawing at 5°C.

As indicated in table V, very little difference in survival was noted throughout 6 months storage between cultures which were not frozen and those that had been frozen and thawed previous to storage at 5°C. This indicated that poor survival to -15°C storage was due mainly to the low temperature of storage rather than to the processes of freezing and thawing.

2. Culture Age

It was shown that C. immitis, grown in the synthetic medium of Roessler et al[1], reaches maximum growth at approximately 10 to 14 days incubation. To determine the effect of various culture ages on survival at 5°C, four series of arthrospore cultures were grown for 7, 14, 21, or 28 days (inoculated on successive weeks to mature on the same day) under standard conditions. Three replicate cultures from each age group (100 ml per culture) were placed in storage at 5°C and assayed for viability at monthly intervals.

As shown in table VI, survival of the 14, 21, and 28 day cultures remained constant (73 to 88 per cent) for the first three months, however, maximum survival was obtained with the 14 day cultures throughout 9 months storage. The 7 day cultures demonstrated a progressive decrease in survival to a 2 per cent level by the third month.

3. Concentration of Cells

Four series of arthrospore cultures were centrifuged and resuspended in their own supernatant liquid to one half, one, two, and four times the original culture volume, respectively, resulting in the cell concentrations shown in table VII. Three replicate cultures (100 ml per culture) of each concentration were placed in storage at 5°C and assayed for viability at monthly intervals.

Survival at all concentrations remained at approximately 100 per cent for the first 5 months. At this point survival of the diluted cultures decreased at a faster rate than that of the more concentrated cultures, and at 18 months the concentrated arthrospore suspension alone gave greater than 1 per cent survival.

4. Volume of Culture Stored

It was noted that storage survival was higher when arthrospore cultures were stored in 100 ml volumes than when stored in 50 ml volumes[4].

This prompted studies of the effects of culture and container volume relationships on storage stability.

a. Varied Culture Volumes in Storage Containers of Identical Capacity

Replicate arthrospore cultures were stored at 5°C in 25, 50, 100, and 200 ml volumes per 8 oz prescription bottle as indicated in table VIII. In general, viability increased directly with culture volume for the 25, 50, and 100 ml volumes throughout 14 months. During the first five months, the viability of the 200 ml cultures was slightly less than that of the 100 ml cultures, suggesting that other factors may have been involved in the different levels of viability.

Varying the culture volume within storage containers of identical capacity also caused a variation of (a) the ratio of the culture surface to culture volume, (b) the ratio of air space to culture volume, (c) the height of the supernatant liquid above the settled cell laye during storage, and (d) the depth of the settled cell layer. Any interpretation of these data must include all of these relationships, and therefore further examination was necessary.

b. Varied Culture Volumes Stored Under Conditions of Constant Ratios of (a) Culture Surface to Culture Volume, and (b) Air Space to Culture Volume

Replicate 25, 50, 100, and 200 ml volumes of arthrospore culture were placed at 5°C storage in 2, 4, 8, and 16 oz prescription bottles, respectively. The various sized bottles had the same general shape, resulting in constant culture surface to culture volume, and air space to culture volume ratios within the storage containers.

As shown in table IX, very little difference in survival was noted among the various volumes of culture, throughout 14 months storage, in contrast to the data in table VIII. This suggested that in the previous experiment the air space within the storage containers might have influenced viability to a greater extent than culture volume, since viability varied directly with an increase in air space to culture volume ratio in the first experiment (table VIII) but remained the same in the second experiment (table IX) when the ratio was the same even though the culture volume was changed.

c. Oxygen and/or Carbon Dioxide Diffusion During Storage

The data from the two previous experiments indicated possible effects on viability due to changes in the gaseous composition above the cultures. These changes might be the result of either shaking or opening the cultures at the monthly plating intervals. This was examined by placing twelve series of replicate cultures (100 ml per 8 oz prescription bottle) in storage at 5°C. Culture series 1 was shaken vigorously and opened at 12 monthly intervals for viability determinations. In contrast, culture series 2 through 8 were shaken and opened only once, each, for plating at the 2nd, 3rd, 4th, 5th, 6th, 9th or 12th monthly interval, respectively. Culture

series 9 through 12 were shaken vigorously at each monthly interval, but opened for plating only at the 3rd, 6th, 9th, or 12th monthly interval, respectively. Viability determinations were made on all cultures at the end of 12 months storage.

As indicated in table X, no significant difference in survival was noted among (a) cultures shaken and opened at each interval, (b) cultures unshaken and unopened, and (c) cultures shaken at each interval but unopened (59, 60, 64 per cent, respectively) at the end of 12 months storage.

B. DRYING

Studies at the Naval Biological Laboratories and the Screening Branch, MB Division indicated very poor survival of liquid grown C. immitis arthrospores to aerosolization (approximately 1 per cent or less). Studies in the Nutrition Branch, MB Division indicated equally poor survival to lyophylization and to drying, alone (< 1 per cent). This information suggested a relationship between the lack of stability to drying and poor aerosol stability. It was postulated that studies on the individual processes involved in lyophylization (drying, freezing, and reduced pressure), with the idea of improvement of survival to each, might solve the problem, or at least greatly improve the stability of C. immitis to aerosolization.

1. Adjuvants

a. Screening of Drying Menstrua

Preliminary experiments with three strains of C. immitis indicated survival to drying of less than 1 per cent. The improvement of survival by resuspending arthrospore suspensions in various menstrua before drying was examined. Arthrospore suspensions of the Cash strain were centrifuged at 2000 rpm for one hour and resuspended in aqueous solutions of glucose, sucrose, lactose, peptone, tryptose, gelatin, sodium caseinate, glycerol, and sodium chloride in the levels recorded in table XI. All cultures were recentrifuged following viability determinations, and the supernatant liquid was removed by aspiration. The resulting packed cells were dried over calcium sulfate in an evacuated desiccator at 5°C for 1 week, resuspended to the original volume in fresh synthetic medium, and counted for viability.

As shown in table XI, carbohydrates enhanced survival to drying to a greater degree than protein substances tested, with 8 per cent glucose resulting in the highest survival (12 per cent as compared with 0.01 per cent for the control cultures).

b. Glucose

Replicate arthrospore cultures were resuspended in 0, 8, 12, 16, 20, and 40 per cent aqueous glucose solutions before drying, in order to determine the optimum concentration of glucose for enhancement of stability to drying. From the data in table XII, it appeared that all of the glucose

concentrations tested resulted in essentially equal survival. It was postulated that the higher survival resulting from resuspension in 40 per cent glucose may have resulted from faulty plate counts due to high viscosity of the glucose solution.

c. Glycerol

Replicate arthrospore cultures were resuspended in 0, 5, 10, 15, 20, and 40 per cent aqueous glycerol solutions before drying. As indicated in table XIII, the use of 5 per cent glycerol resulted in the highest survival to drying (25 per cent as compared with 0.36 per cent for the control cultures). The very low survival resulting from the use of 15 to 40 per cent glycerol may have been due to toxicity of these concentrations.

d. Soviet Menstruum for the Storage of Vaccines

Favorable results from storage of vaccines in a menstruum composed of 15 per cent sucrose, 1.3 per cent gelatin, and 0.1 per cent agar were reported by Soviet scientists[5]. An investigation of this menstruum, pertinent to its value as a pre-drying menstruum for C. immitis was initiated. Replicate arthrospore cultures were resuspended in the Soviet menstruum, in a modification of the menstruum in which 8 per cent glucose was substituted for 15 per cent sucrose, in 8 per cent glucose, in 15 per cent sucrose, and in their own supernatant liquid. Other studies were carried out, to determine optimum levels of the various components of the menstruum, and to evaluate the addition of glycerol or the substitution of glycerol for glucose in the modified menstruum.

As shown in table XIV, highest survival to drying was obtained from Modifications I and II of the Soviet menstruum in which glucose was substituted for sucrose (45 and 49 per cent, respectively, as compared with 37 and 0.31 per cent for the original Soviet menstruum and the control cultures, respectively). Glycerol was found to be inferior to glucose as a substitute for sucrose.

2. Culture Age

Replicate arthrospore cultures incubated for 7, 14, 21, and 28 days under standard conditions (inoculated on successive weeks to mature on the same day) were resuspended in 8 per cent aqueous glucose solution before drying and evaluated as to the effect of age on survival.

As indicated in table XV, higher survival was obtained with either the 14 or the 21 day cultures (29 and 39 per cent, respectively) than with the 7 or 28 day cultures (14 and 1.4 per cent, respectively).

C. FREEZING AND THAWING

1. Physical Studies*

a. Temperature of Freezing and Thawing

Replicate 21 day arthrospore cultures of strain Cash were frozen en masse at -15°C (deep freeze chest) and at -72°C (acetone-dry ice bath). Three cultures from each of the two freezing methods were thawed at 5°C, 25°C, and 37°C, requiring approximately 8, 1, and 0.5 hours, respectively.

As shown in table XVI, survival of cultures frozen at -15°C averaged approximately 40 per cent, as compared with < 1 per cent for cultures frozen at -72°C. Thawing temperature had less effect on survival than temperature of freezing, with slightly higher recoveries at 5°C, than at 25°C or 37°C. Shell freezing at -72°C resulted in equally poor survival as freezing en masse at the same temperature.

Several observations on the freezing process in the two methods indicated that more than one factor may have influenced survival to freezing. It was noted that cultures frozen in the acetone-dry ice bath began freezing immediately, from the periphery toward the center of the container. The samples were completely frozen in approximately 1.5 minutes, with an elevated peak forming in the center of the frozen mass. This indicated possible mechanical stress within the cultures. On the other hand, cultures placed in the deep-freeze chest at -15°C remained in a liquid super-cooled state for approximately 5 hours, at which time snap-freezing occurred in a fraction of a second when the tubes were moved for examination. The surfaces of these cultures were perfectly smooth and level. It appeared that freezing was actually quicker at -15°C, and that physical factors in the freezing process accounted for differences in survival at the two temperatures.

(1) Cell Destruction

It was not possible to establish by microscopic examination whether the freezing and thawing process at -72°C resulted in physical breakdown of cells. However, measurement of cell protein content of the supernatant liquid of cultures before freezing and after thawing indicated cellular destruction. Protein determinations (standard biuret method) were made on the supernatant liquid of 12 replicate 21 day arthrospore cultures. Six cultures were then frozen at -72°C and the other six at -15°C. All cultures were thawed immediately at 5°C and the protein determinations repeated.

As indicated in table XVII, a 20 per cent increase in protein was noted in the supernatant liquid after freezing at -72°C,

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*In some of these studies the arthrospores were resuspended in 8 per cent glucose because of its beneficial effect on survival to drying.

suggesting a release of cell protein upon rupture of the cell wall. This may account, in part, for the very low recovery (0.09 per cent) of spores following freezing at -72°C. No significant change was noted in the protein content of the supernatant liquid of cultures frozen at -15°C. The 34 per cent recovery of spores frozen at the latter temperature suggested that a factor(s), other than rupture of the cell wall, may be involved in poor survival to freezing. This postulation was strengthened further by the fact that loss of viability during freezing at -15°C, but not at -72°C, could be overcome almost completely by resuspension of spores in 8 per cent aqueous glucose before freezing*.

(2) Effect of Temperature

In order to separate the effect of temperature from other factors reducing the viability of cultures frozen at -72°C, replicate 21 day arthrospore cultures were resuspended in (a) their own supernatant liquid and (b) 8 per cent aqueous glucose. Three cultures from each suspension were frozen at -15°C and held at that temperature for one hour. Three additional cultures from each suspension were frozen at -15°C and placed at -72°C (acetone-dry ice bath) for one hour. A third series from each suspension was frozen and held at -72°C for one hour. All cultures were thawed at 5°C and survival determined.

As shown in table XVIII, survival of cultures which were frozen in their own supernatant liquid at -15°C and maintained at either -72°C or -15°C for one hour was 32 to 39 per cent, as compared with < 1 per cent for the cultures which were frozen at -72°C, suggesting that temperature of freezing was more important than the holding temperature in survival to freezing and/or thawing. Similar survival relationships were true for cultures resuspended in 8 per cent glucose before freezing.

b. Repeated Freezing

Replicate 21 day arthrospore cultures were resuspended in (a) their own supernatant liquid and (b) in 8 per cent aqueous glucose solution, frozen at -15°C, and thawed at 5°C. This procedure was repeated a number of times. Viability determinations were made on the original suspensions and after each freezing and thawing cycle.

As shown in table XIX, the greatest loss in viability of cultures frozen in their own supernatant liquid occurred after each of the first two cycles (40 to 50 per cent), thereafter a leveling off occurred with a 3 per cent decrease after each successive cycle. Resuspension in 8 per cent glucose resulted in marked reduction of viability loss so that approximately 50 per cent of the spores survived 5 freeze-thaw cycles.

c. Volume of Cell Suspension

Experiments to determine the effect of repeated freezing and thawing cycles (5 to 8) resulted in a reduction in volume of the 10 ml culture aliquots to 2 to 5 ml due to the removal of successive 1 ml aliquots for viability determinations. To determine whether the diminishing culture volume

*See section C., 2., a., this report.

had any affect on viability to successive freezing and thawing, replicate arthrospore cultures were dispensed in 5, 10, 20, and 40 ml aliquots, twice frozen at -15°C and thawed at 5°C. Survival determinations after each cycle (table XX) indicated approximately 20 per cent higher survival of the 20 and 40 ml aliquots, than that of the 5 and 10 ml aliquots after the first cycle, but no significant difference among the various volumes following the second freezing and thawing cycle.

d. Concentration of Cells

Replicate arthrospore cultures were centrifuged and resuspended in (a) their own supernatant liquid, and (b), (c), and (d) to one half, twice, and four times the original volume of their supernatant liquid, respectively, frozen at -15°C and thawed at 5°C. Very little difference in survival among cultures with concentration levels of from 66 to 750 x 10^6 spores per ml, as indicated in table XXI.

e. Packed Cells Versus Suspended Cells

A comparison of survival to freezing of packed cells (supernatant liquid removed) and suspended cells was made by centrifuging replicate arthrospore cultures, resuspending one half of the cultures in their own supernatant liquid and the other half in 8 per cent glucose. One half the cultures in each of these two groups were recentrifuged and the supernatant liquid removed. Triplicate cultures from each of the resulting four groups (suspensions and packed cells from the culture filtrate and glucose-suspended cultures) were frozen at -15°C and thawed at 5°C.

Survival determinations (table XXII) indicated higher recovery (40 per cent) of packed cells than that (28 per cent) of cells suspended in culture filtrate. However, this relationship was not apparent with cells treated with 8 per cent glucose.

f. Age of Cells

(1) Various Incubation Periods

Replicate arthrospore cultures were grown under standard conditions for 7, 14, 21, and 28 days, and (a) resuspended in their own supernatant liquid, (b) resuspended in 8 per cent aqueous glucose, and (c) diluted 100 fold in 8 per cent glucose. Three cultures from each age group were frozen at -15°C and thawed at 5°C. The freezing and thawing process was repeated a number of times.

As shown in table XXIII, survival decreased with age in cultures suspended in their own supernatant liquid, with a distinct line of demarkation occurring between the 14 and 21 day cultures. This difference was not as pronounced in cultures frozen in the presence of glucose.

(2) Effect of Spent Medium

To investigate the possibility that the presence or absence of some end-product in culture filtrate of young or old cultures produced the differences in the survival shown in table XXIV, replicate cultures of strain Cash were grown for 14 and 28 days. Cultures from each age group were resuspended in (a) their own supernatant liquid, (b) supernatant liquid from the other age group, (c) 8 per cent aqueous glucose solution, and (d) fresh synthetic medium (the same medium in which they were grown). All cultures were frozen at -15°C and thawed at 5°C. The freezing and thawing process was repeated several times.

Several observations were made from the data given in table XXIV: (a) the younger cells were again more stable that the older cells, (b) there was no significant difference in the effect on survival between the 14 and the 28 day old culture filtrates, and (c) the absence of culture filtrate (resuspension in fresh medium) more than doubled the survival in either age group. The latter observation could either have been due to the lack of some toxic end-product or to an increase in the glucose concentration (0.11 M) in the suspending fluid.

2. Effect of Adjuvants

a. Glucose

The optimum level of glucose for increasing survival to freezing was investigated by resuspending replicate cultures of strain Cash in 0, 5, 10, 20, 40, and 80 per cent aqueous glucose solutions, freezing at -15°C and thawing at 5°C.

It was evident from data in table XXV, that glucose improved the survival to freezing at -15°C (and thawing at 5°C), and that there was little difference among the 5 to 40 per cent levels. It was postulated that the inconsistent results in experiments I and II were due to (a) poor dispersion of cells throughout the glucose solutions (because of the viscosity of the higher levels of glucose) and/or (b) the resulting loss of cells adhering to the inside walls of pipettes. In an attempt to eliminate these factors in experiment III, the cultures were shaken from 5 to 10 minutes on a Kahn shaker and the pipettes used in making the first dilutions for plating were rinsed out in the diluting fluid three times. As shown by the results, a uniform survival of approximately 100 per cent was obtained in experiment III throughout the range of 5 to 40 per cent glucose solution. Similarly, the control cultures in experiment III were higher than those in the first two experiments.

b. Glycerol

The optimum level of glycerol for increasing survival to freezing was investigated by resuspending replicate arthrospore cultures in 0, 5, 8, 10, 20, 30, 40, and 80 per cent aqueous glycerol, freezing at -15°C and thawing at 5°C. As shown in table XXVI, all concentrations of glycerol resulted in an increase in survival to freezing and thawing, with 20 per cent as the optimum (83 per cent as compared with 36 per cent for the controls).

c. Combinations of Glycose and Glycerol

An investigation of the combined effects on survival to freezing and thawing of glucose and glycerol was made by preparing suspending menstrua containing the various levels of these two compounds shown in table XXVII. Stock solutions of each compound previously showing maximum protective effect (8 per cent glucose and 20 per cent glycerol) were combined in glucose:glycerol proportions of 10:0, 9:1, 8:2, etc. Replicate arthrospore cultures were resuspended in each of the 11 menstrua, frozen at -15°C and thawed at 5°C.

The protective effects of the two compounds were not additive. Survival of cells in the various menstrua was not greatly different, averaging approximately 90 per cent, as compared with 37 per cent for the controls.

TABLE I. EFFECT OF TEMPERATURE, GROWTH MEDIUM, AND SUPPLEMENT ON SURVIVAL OF CASH AND M-11 STRAINS OF C. *LIMITIS* TO STORAGE

STRAIN, MEDIUM AND TREATMENT	PER CENT VIABILITY[a]					
	1 Mo.		2 Mo.		3 Mo.	
	5°C	25°C	5°C	25°C	5°C	25°C
Cash - Synthetic						
Control	101	104	106	93	65	14
Fresh Medium	118	134	122	47	56	25
+ Glucose[b]	112	109	102	110	76	52
+ Yeast - 75[c]	104	107	98	59	55	16
Cash - Complex						
Control	47	< 1	27	—	11	—
Fresh Medium	96	< 1	44	—	3	—
+ Glucose[b]	63	< 1	39	—	16	—
+ Yeast - 75[c]	71	< 1	14	—	2	—
M-11 - Synthetic						
Control	93	80	100	62	67	36
Fresh Medium	112	97	82	63	72	10
+ Glucose[b]	95	104	86	107	89	95
+ Yeast - 75[c]	72	67	58	39	53	16
M-11 - Complex						
Control	78	13	70	1	58	—
Fresh Medium	111	26	81	< 1	62	—
+ Glucose[b]	65	13	51	< 1	49	—
+ Yeast - 75[c]	85	20	68	< 1	34	—

a. Mean values of three replicate cultures.
b. Sufficient glucose solution added to culture to bring glucose concentration to 2 per cent.
c. Sufficient Yeast 75 solution added to culture to bring Yeast 75 concentration to 0.25 per cent.

TABLE II. EFFECT OF PRE-STORAGE SUPPLEMENTATION ON SURVIVAL OF C. IMMITIS AT 5°C.

SUPPLEMENT	PER CENT VIABILITY[a] Months Stored								
	1	3	5	7	9	10	11	12	13
None (control)	99 (87-107)	100 (96-132)	107 (97-117)	105 (96-110)	108 (98-119)	100 (84-108)	77 (59-92)	53 (48-58)	19 (16-21)
Nitrogen Source	112 (106-123)	126 (114-135)	126 (106-140)	105 (87-120)	113 (91-130)	105 (78-126)	85 (78-93)	73 (57-87)	33 (0.9-88)
Carbon Source[c]	113 (103-129)	133 (115-154)	130 (123-135)	120 (110-126)	134 (126-146)	124 (120-127)	101 (76-129)	84 (64-106)	60 (39-88)
Nitrogen source + Carbon source	122 (99-136)	139 (129-152)	142 (121-156)	150 (124-174)	152 (136-161)	166 (156-176)	144 (137-151)	134 (122-151)	127 (103-137)

a. Mean values of three replicate cultures. Figures in parthentheses indicate upper and lower limits.
b. 0.08 M ammonium acetate.
c. 0.11 M glucose.

TABLE III. EFFECT OF DRYING OR RESUSPENDING ARTHROSPORES IN 8 PER CENT GLUCOSE BEFORE STORAGE ON SURVIVAL AT 5°C.

TREATMENT	ORIGINAL COUNT	POST DRYING COUNT	VIABILITY[a]						
			1 Mo.	2 Mo.	3 Mo.	4 Mo.	5 Mo.	6 Mo.	7 Mo.
	x 10^6	x 10^6	%	%	%	%	%	%	%
Controls	224	—	103	110	115	96	91	126	119
Undried, stored in 8% Glucose.	190	—	89	112	120	92	107	113	117
Dried in 8% Glucose, then resuspended and stored in 8% glucose.	—	96[b]	102	98	75	42	41	33	32

a. Mean values of three replicate cultures.
b. Used as a base line of 100 per cent.

TABLE IV. SURVIVAL OF C. INSOLITUS AT -15°C.

PRE-FREEZING SUSPENDING MENSTRUUM	COUNT AFTER FREEZING AND THAWING	PER CENT VIABILITY[a]									
		Months Stored									
	x 10^6	1	2	3	4	5	6	7	8	9	10
Culture Filtrate	66	46	27	21	20	33	14	23	10	7	7
8 Per Cent Glucose	316	77	77	61	68	77	53	63	53	35	35

a. Mean values of three replicate cultures.

TABLE V. EFFECT OF FREEZING ON SURVIVAL OF C. INNITIS AT 5°C.

TREATMENT	ORIGINAL COUNT	POST FREEZING COUNT	PER CENT VIABILITY[a] Months Stored								
			1	2	3	4	5	6	7	8	9
	x 10^6	x 10^6									
Control	224	—	103	110	115	96	91	126	119	111	89
Resuspended in 8% Glucose	190	—	89	112	120	92	107	113	117	125	112
Resuspended in 8% Glucose, Frozen at -15°C, and Thawed at 5°C	—	154[b]	81	99	103	88	94	92	87	86	86

a. Mean values of three replicate cultures.
b. Used as 100 per cent base line (for further viability determinations).

TABLE VI. EFFECT OF CULTURE AGE ON SURVIVAL OF C. IMMITIS AT 5°C.

CULTURE AGE	ORIGINAL COUNT	PER CENT VIABILITY[a]				
		1 Mo.	2 Mo.	3 Mo.	6 Mo.	9 Mo.
days	x 10^6					
28	344	75	73	73	52	37
21	316	85	88	82	41	23
14	314	84	88	82	72	51
7	250	72	33	2	< 1	< 1

a. Mean values of three replicate cultures.

TABLE VII. EFFECT OF CELL CONCENTRATION ON SURVIVAL OF C. IMMITIS AT 5°C.

CELL CONCENTRATION	PER CENT VIABILITY[a] Months Stored											
	1	2	3	4	5	6	8	10	12	14	16	18
x 10^6												
730	97	92	104	106	95	96	70	53	38	32	18	11
305	103	100	74[b]	100	94	100	75	50	12	3	1	< 1
162	114	104	103	100	94	85	59	43	5	1	< 1	< 1
86	102	113	112	120	102	88	58	27	12	4	2	< 1

a. Mean values of three replicate cultures.
b. Questionable counts (plates contaminated).

TABLE VIII. EFFECT OF CULTURE VOLUME ON SURVIVAL OF C. IMMITIS AT 5°C.

VOLUME OF STORED CULTURE	ORIGINAL COUNT	PER CENT VIABILITY[a]								RATIOS WITHIN STORAGE CONTAINER	
		1 Mo.	2 Mo.	3 Mo.	4 Mo.	5 Mo.	6 Mo.	10 Mo.	14 Mo.	C.[b] Surface / C. Volume	Air Space / C. Volume
ml	x 10^6										
25	274	96	99	69	62	54	44	19	2	1.3	9
50	284	104	104	84	78	71	68	73	49	0.65	4
100	283	130	113	102	89	89	88	83	67	0.32	1.5
200	276	118	102	86	86	84	90	85	75	0.11	0.25

a. Mean values of three replicate cultures.
b. C. = Culture.

TABLE IX. EFFECT OF VOLUME OF CULTURE AT CONSTANT ($\frac{CS}{CV}$) AND ($\frac{AS}{CV}$) RATIOS ON SURVIVAL OF C. LIMITIS AT 5°C.[a]

VOLUME OF STORAGE CONTAINER	VOLUME OF CULTURE STORED	ORIGINAL COUNT	PER CENT VIABILITY[b]							
			1 Mo.	2 Mo.	3 Mo.	4 Mo.	5 Mo.	6 Mo.	10 Mo.	14 Mo.
oz	ml.	x 10^6								
2	25	373	84	91	84	70	71	67	65	47
4	50	325	109	103	102	91	99	87	81	60
8	100	361	100	111	114	121	106	85	78	49
16	200	344	95	105	99	97	99	87	70	59

a. Culture surface to culture volume ($\frac{CS}{CV}$) and air space to culture volume ($\frac{AS}{CV}$) ratios constant.

b. Mean values of three replicate cultures.

TABLE X. EFFECT OF SHAKING AND/OR OPENING STORAGE CONTAINERS DURING STORAGE ON SURVIVAL OF C. INNOCUIS AT 5°C.

CULTURE SERIES	TREATMENT	PER CENT VIABILITY[a]												
		1 Mo	2 Mo	3 Mo	4 Mo	5 Mo	6 Mo	7 Mo	8 Mo	9 Mo	10 Mo	11 Mo	12 Mo	Average at 12 Months
1	Shaken and Opened at Monthly Intervals	83	79	82	88	57[b]	86	85	76	71	73	59	59	59
2	Unshaken and Unopened, Except at Specified Intervals	→	83	→	→	→	→	→	→	→	→	→	48	65
3		→	→	85	→	→	→	→	→	→	→	→	66	
4		→	→	→	96	→	→	→	→	→	→	→	61	
5		→	→	→	→	82	→	→	→	→	→	→	70	
6		→	→	→	→	→	101	→	→	→	→	→	82	
7		→	→	→	→	→	→	→	→	82	→	→	70	
8		→	→	→	→	→	→	→	→	→	→	→	60	
9	Shaken at Each Monthly Interval, but Unopened Except at Specified Intervals	→	→	92	→	→	→	→	→	→	→	→	66	60
10		→	→	→	→	→	82	→	→	→	→	→	55	
11		→	→	→	→	→	→	→	→	77	→	→	64	
12		→	→	→	→	→	→	→	→	→	→	→	54	

a. Mean values of three replicate cultures.
b. Low counts due to contaminated plates.

TABLE XI. EFFECT OF DRYING MENSTRUUM ON SURVIVAL OF *C. TENUIS* TO DRYING.

DRYING MENSTRUUM[a]	VIABLE COUNT[b]		SURVIVAL
	Predrying	Postdrying	
	x 10^6	x 10^6	%
8% Glucose	240	28	12
4.5% Glucose	304	25	8.2
8% Sucrose	292	14	4.8
4.5% Sucrose	241	9.4	5.4
8% Lactose	287	3.4	1.2
4% Lactose	282	1.6	0.6
10% Sodium Caseinate	184	0.2	0.09
5% Sodium Caseinate	179	6.3	3.5
5% Peptone	268	0.2	0.08
1% Peptone	289	0.2	0.07
5% Gelatin	249	0.4	0.14
1% Gelatin	151	0.2	0.43
0.85% NaCl	270	0.1	0.04
Saturated NaCl	146	0.1	0.04
Tryptose Saline Diluent[c]	282	0.9	0.31
Synthetic Medium[d]	285	0.5	0.16
5% Glycerol	283	8.9	3.1
Culture Filtrate (Control)	285	0.27	0.01

a. Centrifuged growth resuspended in the menstruum, recentrifuged, and the supernatant liquid removed before drying.
b. Mean values of 3–6 cultures (5 experiments).
c. 0.1 per cent tryptose, 0.5 per cent NaCl.
d. Glucose, ammonium acetate, inorganic salts medium.

TABLE XII. EFFECT OF RESUSPENSION IN GLUCOSE ON SURVIVAL OF C. DIMITIS TO DRYING.

DRYING MENSTRUUM	VIABLE COUNT[a]		SURVIVAL
	Predrying	Postdrying	
% Glucose	x 10^4	x 10^4	%
0	286	0.23	0.08
8	309	151	50
12	308	176	57
16	315	172	55
20	316	164	52
40	239	166	69

a. Mean values of duplicate cultures.

TABLE XIII. EFFECT OF RESUSPENSION IN GLYCEROL ON SURVIVAL OF S. IMMITIS TO DRYING.

DRYING MENSTRUUM	VIABLE COUNT[a]		SURVIVAL
	Predrying	Postdrying	
% Glycerol	x 10^6	x 10^6	%
0	290	10	0.36
5	256	64	25
10	300	35	12
15	238	6	3
20	168	4	2
40	132	1	0.7

a. Mean values of three replicate cultures.

TABLE XIV. EFFECT OF RESUSPENSION IN SOVIET MENSTRUUM ON SURVIVAL OF C. IMMITIS TO DRYING.

DRYING MENSTRUUM		VIABLE COUNT[a]		SURVIVAL
		Predrying	Postdrying	
		x 10^6	x 10^6	%
Culture Filtrate		272	0.85	0.31
Soviet Menstruum[b]		300	110	37
Modification I[c]		292	131	45
Modification II[d]		314	155	49
Modification III[e]		254	21	8
Modification IV[f]		236	92	39
Glucose	8%	290	105	36
Sucrose	15%	278	53	19
Glycerol	5%	280	64	23
Gelatin	0.1%	198	0.75	0.38
Gelatin	1.3%	252	3.7	1.50
Gelatin	2.5%	208	1.31	0.63
Agar	0.1%	336	0.83	0.25
Agar	0.2%	284	65	23[g]
Agar	0.3%	302	50	17[g]

a. Mean values of 3 to 18 cultures, in 6 separate experiments.
b. 15 per cent sucrose, 1.3 per cent gelatin, and 0.1 per cent agar.
c. 8 per cent glucose, 1.3 per cent gelatin, and 0.1 per cent agar.
d. 8 per cent glucose, 0.7 per cent gelatin, and 0.05 per cent agar.
e. 5 per cent glycerol, 1.3 per cent gelatin, and 0.1 per cent agar.
f. 8 per cent glucose, 5 per cent glycerol, 1.3 per cent gelatin, and 0.1 per cent agar.
g. 0.2 and 0.3 per cent did not permit drying of these cultures, thus resulting in the high survival in these cultures.

TABLE XV. EFFECT OF CULTURE AGE ON SURVIVAL OF C. LIMITIS TO DRYING.

AGE OF CULTURE	VIABLE COUNT[a]		SURVIVAL
	Predrying	Postdrying	
days	x 10^4	x 10^4	%
28	190	2.6	1.4
21	236	92	39
14	272	78	29
7	140	20	14

a. Mean values of duplicate cultures suspended in 8 per cent glucose.

TABLE XVI. EFFECT OF FREEZING AND THAWING ON SURVIVAL OF C. IMMITIS

METHOD OF FREEZING	METHOD OF THAWING	VIABLE COUNT[a]		SURVIVAL
		Prefreezing	Post-thawing	
		x 10^6	x 10^6	%
Acetone,[b]	Cold-Room (5°C)	300	0.53	0.2
Dry-Ice	Incubator (25°C)	300	0.06	0.02
Bath (-72°C)	Incubator (37.5°C)	300	0.05	0.02
Deep	Cold-Room (5°C)	274	142	52
Freeze[b]	Incubator (25°C)	274	100	35
(-15°C)	Incubator (37.5°C)	274	108	40

a. Mean values of triplicate aliquots.
b. Frozen en-masse (not shell frozen).

TABLE XVII. EFFECT OF TEMPERATURE OF FREEZING ON RELEASE OF CELL PROTEIN BY C. [illegible]

FREEZING TEMPERATURE	PROTEIN IN SUPERNATANT LIQUID[a]			SURVIVAL
	Prefreezing	Postfreezing	Increase	
C	mg/ml	mg/ml	%	%
-72°	1.56	1.88	20.5	0.09
-15°	1.53	1.54	0.65	34

a. Mean values of six samples.

TABLE XVIII. EFFECT OF TEMPERATURE OF FREEZING ON SURVIVAL OF C. DERMITIS.

TREATMENT	FREEZING MENSTRUUM	VIABLE COUNT[a]		SURVIVAL
		Prefreezing	Postfreezing	
		x 10^6	x 10^6	%
Frozen and Held for 1 Hour at -15°C	Culture Filtrate	410	158	39
	8 Per cent Glucose	372	302	81
Frozen at -15°C and Held for 1 Hour at -72°C	Culture Filtrate	444	142	32
	8 Per cent Glucose	396	246	62
Frozen and Held for 1 Hour at -72°C	Culture Filtrate	326	0.06	0.02
	8 Per cent Glucose	314	0.38	0.12

a. Mean values for three replicate cultures.

TABLE XIX. SURVIVAL OF C. DERMATIS TO REPEATED FREEZING (-15°C) AND THAWING (5°C).

FREEZING MENSTRUUM	ORIGINAL COUNT	NUMBER OF FREEZING AND THAWING CYCLES[a]				
		1	2	3	4	5
	x 10^6	%	%	%	%	%
Culture Filtrate	290	50	10	7	4	0.6
8 Per Cent Glucose	250	93	72	65	58	43

a. Mean survival values of three replicate cultures.

TABLE XX. EFFECT OF CULTURE VOLUME ON SURVIVAL OF C. DIMITIS

VOLUME OF ALIQUOT	ORIGINAL COUNT	NO. OF FREEZING AND THAWING CYCLES[a]	
		1	2
ml	x 10^6	%	%
5	356	43	25
10	358	43	25
20	328	62	22
40	312	61	22

a. Mean survival values for three replicate cultures.

TABLE XXI. EFFECT OF CELL CONCENTRATION ON SURVIVAL OF C. INNOCUA TO FREEZING (-15°C) AND THAWING (5°C).

CELL CONCENTRATION	VIABLE COUNT[a]		SURVIVAL
	Prefreezing	Postfreezing	
	x 10^6	x 10^6	%
2X	750	206	28
1X	272	94	35
½X	134	39	29
¼X	66	18	27

a. Mean values of three replicate cultures.

TABLE XXII. COMPARISON OF SURVIVAL OF PACKED CELLS AND SUSPENDED CELLS OF C. DIPHTHIS TO FREEZING (-15°C) AND THAWING (5°C).

FREEZING MENSTRUUM	CONDITION OF CELLS	VIABLE COUNT[a]		SURVIVAL
		Prefreezing	Postfreezing	
		x 10^6	x 10^6	%
Culture Filtrate	Suspended	230	65	28
	Packed (Filtrate removed)	264	107	40
8 Per Cent Glucose	Suspended	258	216	84
	Packed (Glucose solution removed)	254	184	72

a. Mean values for three replicate cultures.

TABLE XXIII. EFFECT OF AGE OF CELLS ON SURVIVAL OF C. DERMITIS TO FREEZING (-15°C) AND THAWING (5°C).

FREEZING MENSTRUUM	AGE OF CELLS	ORIGINAL COUNT	NO. OF FREEZING AND THAWING CYCLES[a]				
			1	2	3	4	5
	days	x 10^6	%	%	%	%	%
Culture Filtrate	7	335	53	19	8	3	0.8
	14	320	41	18	11	8	2
	21	328	18	6	1	0.5	0.3
	28	370	11	3	1	0.3	—
Culture Diluted 100-Fold in 8% Glucose	7	2.89	45	39	33	24	19
	14	3.10	75	56	51	46	36
	21	3.64	56	47	34	28	28
	28	2.96	50	41	33	30	—
Culture Resuspended in 8% Glucose	14	304	84	59	60	56	44
	21	320	61	53	36	36	36
	28	320	45	38	31	29	—

a. Mean survival values for three replicate cultures.

TABLE XXIV. EFFECT OF CULTURE AGE ON SURVIVAL OF C. DIMITIS TO FREEZING (-15°C) AND THAWING (5°C).

AGE OF CELLS	FREEZING MENSTRUUM	ORIGINAL COUNT	NO. OF FREEZING AND THAWING CYCLES[a]			
			1	2	3	4
days		x 10^6	%	%	%	%
14	14 Day Culture Filtrate	256	26	9	3	2
	28 Day Culture Filtrate	264	27	11	3	2
	8 Per Cent Glucose	282	85	63	29	22
	Fresh Medium	276	51	23	5	3
28	28 Day Culture Filtrate	274	20	5	1	0.6
	14 Day Culture Filtrate	218	17	3	0.5	0.3
	8 Per Cent Glucose	232	66	45	32	28
	Fresh Medium	222	56	28	17	12

a. Mean survival values for three replicate cultures.

TABLE XXV. EFFECT OF RESUSPENSION IN AQUEOUS GLUCOSE ON SURVIVAL OF C. INITIS TO FREEZING (-15°C) AND THAWING (5°C).

FREEZING MENSTRUUM	EXP. I[a]		EXP. II[a]		EXP. III[a]		AVERAGE SURVIVAL
	Original Count	Survival	Original Count	Survival	Original Count	Survival	
% Glucose	x 10^6	%	x 10^6	%	x 10^6	%	%
0	252	32	304	63	372	68	54
5	226	93	268	90	322	100	94
10	220	82	244	98	350	100	93
20	200	81	248	86	342	99	89
40	100	104	86	88	290	101	98
80	76	53	74	41	220	52	49

a. Mean values of three replicate cultures.

TABLE XXVI. EFFECT OF GLYCEROL ON SURVIVAL OF C. DERMITIS TO FREEZING (-15°C) AND THAWING (5°C).

FREEZING MENSTRUUM	ORIGINAL COUNT[a]	SURVIVAL[a]	AVERAGE SURVIVAL
% Glycerol	x 10^6	%	%
0	280, 258, 342	34, 37, 36	36
5	332	68	68
8	260, 262	62, 63	63
10	328	75	75
20	112, 152, 304	84, 84, 81	83
30	312	81	81
40	70, 114, 208	46, 69, 66	60
80	250	6	6

a. Values from separate experiments. Each value represents the mean of 3 samples.

TABLE XXVII. COMBINED EFFECT OF GLUCOSE AND GLYCEROL ON SURVIVAL OF C. *BOVITIS* TO FREEZING (-15°C) AND THAWING (5°C).

FREEZING MENSTRUUM		VIABLE COUNT		SURVIVAL
Glycerol	Glucose	Prefreezing	Postfreezing	
%	%	x 10^6	x 10^6	%
20	0	224	212	95
18	0.8	258	192	74
16	1.6	252	218	87
14	2.4	250	208	83
12	3.2	286	242	85
10	4.0	290	246	85
8	4.8	266	262	99
6	5.6	310	268	87
4	6.4	324	274	85
2	7.2	302	282	93
0	8.0	282	298	106
0	0	310	116	37

V. CONCLUSIONS

The following general conclusions may be stated from the results of this study:

1. Optimum storage stability at 5°C was obtained with 14-day old arthrospores supplemented with glucose and ammonium acetate or harvested from synthetic medium and resuspended in 8 per cent glucose.

2. The ratio of the air space to culture volume within the storage container was critical, with best stability obtained at a ratio of 1, or less than 1.

3. Disturbance of the storage containers (i.e., shaking or opening) during storage had no effect on viability.

4. Resuspension of arthrospores in 8% glucose markedly increased survival to drying, freezing and thawing, and aerosolization.

5. During the freezing and thawing cycle, the temperature of freezing was more critical for survival than the temperature of thawing.

VI. LITERATURE CITATIONS

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